Culture-Independent Analysis of Midgut Microbiota in the Arbovirus Vector *Culicoides sonorensis* (Diptera: Ceratopogonidae)

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ABSTRACT Differences in midgut microbial communities inhabiting *Culicoides* spp., insect vectors of virus pathogens, may affect the variation observed in the ability of these biting midges to propagate arthropod-borne viruses. As a first step toward addressing this hypothesis, midgut bacterial communities were compared between *Culicoides* species expected to be efficient and inefficient vectors of virus pathogens. We used 16S rDNA sequence and restriction fragment information to provisionally identify 36 bacterial genera from guts of wild adult female biting midges, *Culicoides sonorensis* Wirth and Jones and *Culicoides variipennis* (Coquillet), from two geographical locations. Bacterial identification was made by sequence analysis of 16S rDNA fragments and by terminal restriction fragment length polymorphism analysis of polymerase chain reaction-amplified 16S rDNA fragments from adult guts. Of 36 bacterial genera identified, 12 had been previously identified in other insects: *Comomonas*, *Enterobacter*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, *Staphylococcus*, *Chryseobacterium*, *Moraxella*, *Acholeplasma*, *Flavobacterium*, and *Rickettsia*. Significant differences in bacterial community composition were found between all three groups of wild adult females analyzed: live-trapped *C. sonorensis*, laboratory-emerged *C. sonorensis*, and laboratory-emerged *C. variipennis*.

KEY WORDS midgut microbiota, biting midge, arbovirus vector

CULICOIDES SPP. BITING MIDGES transmit two notable agriculturally significant pathogens in the United States, both in the genus Orbivirus: bluetongue virus and epizootic hemorrhagic disease virus. Worldwide, *Culicoides* spp. transmit a variety of viral (arbovirus) pathogens to animals and humans, as well as protozoa and filarial pathogens to birds, humans, and other mammals (reviewed in Mellor et al. 2000). The ability of *Culicoides* spp. to transmit viral pathogens has been variously described as being due to genetic or environmental factors (Tabachnick 1990, 1991; Venter et al. 1998; Fu et al. 1999; Wittmann et al. 2002). Recent studies of environmental factors as determinants of vector competence cited two possible ways in which adult infection rates may vary: 1) changes in larval rearing temperatures and 2) changes in postvirus meal holding temperatures (Mellor et al. 1998, Wittmann et al. 2002). The larval stages of *Culicoides* spp. rely on aquatic microbiota as a nutrient source (Hunt 1994), and, in the wild, occur in aquatic habitats that may be polluted with livestock waste. Constituents of larval midgut microbiotic communities might vary depending on factors such as ambient temperature, local soil type, salinity, and climate (Schmidtmann et al. 2000). In addition, microbiota, including rickettsia-like organisms (Hertig and Wolbach 1924) and protozoa (Hommel and Croft 1975), inhabit the alimentary tract of the adult stage of *Culicoides* spp., as well as other hematophagous dipterans (Vasanthi and Hoti 1992, Demaio et al. 1996, Ignatova et al. 1996).

Other studies have implicated gut bacteria in the modulation of trypanosome or plasmodium propagation in vector insects (Seitz et al. 1987, Maudlin et al. 1990, Pumpuni et al. 1996). Because variations in larval rearing temperature later affect the ability of *Culicoides* adults to become infected with an arbovirus, perhaps the midge midgut microbial community plays a role in this phenomenon. This study presents the first steps toward addressing this hypothesis for arbovirus infection of *Culicoides* spp.

Two closely related midge species, *Culicoides sonorensis* Wirth and Jones and *Culicoides variipennis* (Coquillet), vary in their ability to transmit arboviruses. *C. sonorensis* is an established vector of orbiviruses, whereas *C. variipennis* is a closely related midge with an overlapping distribution area that is an inefficient vector of these viruses (Walton et al. 1992). These two species were considered subspecies before recent elevation to species status (Holbrook et al. 2000). Even within a given species, arbovirus infection rates vary among populations and individuals (Walton et al. 1992). Comparing differences in midgut microbial communities between midge species and between different geographical regions provides a foundation for

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future work to determine the potential role midgut microbial communities might play in vector competence. Any variations in midgut bacteria identified in this pilot study must be analyzed further in subsequent experiments to distinguish between insect population-specific and -habitat differences, as well as to correlate gut microbiota differences to variations in arbovirus infection rates.

It has been estimated that as many as 99% of all microorganisms are not cultivable by standard methods (Hugenholtz et al. 1998). Therefore, we chose analysis of 16S rDNA fragments to assess microbial diversity in *Culicoides* midguts. Terminal-restriction fragment-length polymorphism (T-RFLP) analysis of 16S rDNA amplified from mixed community genomic templates provides a powerful way to compare differences between complex microbial communities (Liu et al. 1997, Kaplan et al. 2001). In this study, T-RFLP analysis was used to determine overall differences in midgut bacterial populations between the insect groups tested, whereas cloning and sequencing of bacterial 16S rDNA fragments provided preliminary bacterial genera assignments.

Both colony-reared *C. sonorensis* and wild *Culicoides* spp. populations were analyzed. As a practical tool to assess the microbial community of colony-reared midges, we used T-RFLP to track any microbial population changes in adult *C. sonorensis* after a blood meal, as has been described for mosquitoes (Demaio et al. 1996). However, the major goal of this study was to delineate the differences in bacterial community composition between a putative arbovirus-susceptible population, wild *C. sonorensis* from a bluetongue endemic region of central Colorado (site 1) and a putative incompetent population, *C. variipennis* from site 2, a location in eastern Nebraska in a transitional zone between endemic and nonendemic regions (Barber 1979). To accomplish this, bacterial 16S rDNA from these two wild midge populations was amplified by polymerase chain reaction (PCR) and either 1) cloned and sequenced or 2) assessed by T-RFLP analysis. These analyses showed significant differences in bacterial populations among the insect populations tested and resulted in the provisional identification of 36 bacterial genera, one-third of which have been isolated from other insect species (Hertig and Wolbach 1924, Tully et al. 1987, Maudlin et al. 1990, Thanabalu et al. 1992, Vasanthi and Hoti 1992, Demaio et al. 1996, Sancho et al. 1996, Hurst et al. 1999, Dugas et al. 2001, Fouda et al. 2001, Perira de Oliveira et al. 2001, Marchini et al. 2002, Wolf et al. 2002).

Materials and Methods

Colony Insects. *C. sonorensis* were reared at the colony maintained at the Arthropod-borne Animal Diseases Research Laboratory, Laramie, WY. Colony midges of the Ausman strain originated from field specimens collected in 2000 from site one near Brighton, CO; this site was also used for collecting one wild midge population used in this study. Two- to 3-d-old midges were fed a meal of defibrinated sheep blood through an artificial membrane feeding apparatus (Hunt 1994) and maintained at 27°C in rearing cages with sterile 10% sucrose. Midguts were dissected from 2- to 3-d-old unfed midges and from blood-fed midges at 1, 2, 3, and 5 d after a blood meal. Midguts were dissected, using sterile technique, in nucleic acid stabilizing solution, RNAlater (Ambion, Austin, TX). Midguts were stored in RNAlater at −20C.

Wild Insects. *C. sonorensis* were collected as either pupae or adults from field site 1 (N 40.01389, W 104.91333) near Brighton, CO. *C. variipennis* were collected from site 2 (N 40.71383, W 95.91547) near Nebraska City, NE. Pupae were sorted at the laboratory into rearing cages and were allowed to emerge as adults. Midguts were removed at 1 d postemergence and stored in RNA later at −20C.

DNA Isolation and Cloning. Genomic DNA isolations (MoBio, Carlsbad, CA) were performed on pools of five midguts each: samples F1 (three midguts), F2, and F3 from site 1, and F29, F30, and F31 from site 2. Genomic DNA yields were in the range of 2–8 ng/μl per sample pool. 16S rRNA fragments were PCR amplified using the methods of Kaplan et al. 2001). PCR primers were as follows: 16SF46, GCY TAA CAC ATG CAA GTC GA; and 16SR536, GTA TTA CCG CGG CTG CTG G. PCR fragments of ≈500 bp were gel purified, cloned into the pCR-TOPO 4.0 sequencing plasmid (Invitrogen, Carlsbad, CA), transformed into *Escherichia coli* and colony purified. Tentative 16S rDNA inserts were sequenced using T3, T7, M13 forward, or M13 reverse sequencing primers on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA) with dRhodamine Ready Reaction cycle sequencing reagent (Applied Biosystems).

Sequence Analysis. Sequences were assembled using Vector NTI software (Informax, Frederick, MD) and analyzed for similarity to known sequences using BLASTn (Altschul et al. 1990). All cloned sequences were screened for the presence of chimeric artifacts using the CHIMERA_CHECK program (version 2.7) of the Ribosomal Database Project (Maidak et al. 2001). Putative chimeric fragments were excluded from further analysis. Phylogenetic analyses used the ARB software program and database (neighbor-joining with Olsen correction) (Ludwig et al. 1998) or GCG Wisconsin package. Cluster names were designated according to the first DNA library clone containing the sequence of interest. For example, in C1–4, first character (i.e., C) denotes collection site, second character (i.e., 1) denotes midgut pool number, and the remaining characters denote the clone number (i.e., 4).

Terminal-Restriction Fragment-Length Polymorphism Analysis. Approximately 25 ng of PCR product was digested with 6 U of *Hha*I, *Rsa*I, or *Alu*I (New England Biolabs, Beverly, MA) for 2 h in the manufacturer's recommended reaction buffer. Digests were purified by passage through gel filtration cartridges (Edge Biosystems, Gaithersburg, MD) and lyophilized. Each sample was then resuspended in 2 μl of deionized formamide, 0.5 μl of loading buffer, and

0.3 μl of Genescan 500 ROX size standards (Applied Biosystems), denatured at 96°C for 2 min, and held on ice. Samples were analyzed by electrophoresis on 5% polyacrylamide gels (6 M urea) by using an ABI 377 DNA sequencer. T-RF sizes between 50 and 500 bp were determined using Genescan analytical software (Applied Biosystems). To quantify electropherogram output, a baseline threshold of 50 fluorescence units, local southern size matching, and heavy smoothing were used.

Comparison of T-RFLP profiles from different samples requires standardization of relative fluorescence between samples (Dunbar et al. 2001). Briefly, relative fluorescence was standardized to the smallest quantity by proportionally reducing each peak area in larger profiles. After proportional reduction of larger profiles, peaks having fluorescence values less than the threshold value were eliminated from subsequent analyses.

To gain insights into compositional changes between samples, T-RF lengths predicted from cloned sequences were compared with T-RF sizes observed in T-restriction fragment-length polymorphism profiles. A difficulty with using T-RF sizes predicted from sequence data to identify T-RF peaks from environmental samples is that observed length and predicted length do not always match. Although we found that predicted lengths for five cloned sequences were all within 2 bp of observed T-RF length, several studies (Gonzalez et al. 2000, Kaplan et al. 2001, Kitts 2001) have found larger (up to 7 bp), apparently sequence-specific, discrepancies between anticipated and observed lengths. Although larger discrepancies are possible, we chose a 2 bp window for increased stringency.

Results

Colonized Midge Gut Bacterial Community Composition Varies after a Blood Meal. Colony *C. sonorensis* adult females were fed a blood meal and held for 1, 2, 3, or 5 d in rearing cages with sterile sucrose as a food source. At selected intervals postfeeding, midguts were removed for genomic DNA isolation and subsequent PCR amplification of 16S rDNA fragments. After digestion with the restriction enzyme *Hha*I, T-RFLP indicated a shift in microbial community composition in the days after the blood meal (Fig. 1); changes in T-RFLP profiles are indicated by variation in the pattern of black boxes over time. A Mantel test (Mantel 1967, Mantel and Vanland 1970, Bonnet and Van de Peer 2002) of Jaccard similarity values and temporal distances (0–5 d) produced an observed correlation (−0.298) and associated *P* value (<0.001) that strongly support this supposition.

Wild *Culicoides* Adult Females: 16S rDNA Cloning, Sequencing, and Analysis. Two groups of wild *Culicoides* pupae, *C. sonorensis* (site 1, Colorado [CO]), and *C. variipennis* (site 2, Nebraska [NE]), were collected from separate sites in the central western United States. Pupae were transferred to rearing cages, and midguts were removed from adults one day posteclosion. After PCR amplification and ligation into plasmids, at least 20 independent bacterial 16S rDNA inserts per midgut pool were sequenced and clustered by neighbor-joining analysis (data not shown) to determine phylogenetic affiliation. Table 1 and Table 2 display these groupings, along with the most highly similar known bacterial genus (BLASTn) (Altschul et al. 1990) and the predicted bacterial 16S rDNA T-RF sizes.

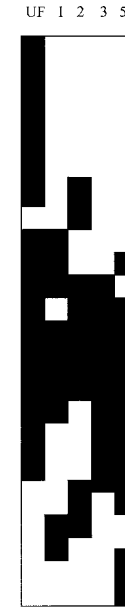

Fig. 1. Colonized midge midgut bacterial community composition changes after blood meal. Presence/absence of T-RFs after *Hha*I digest of whole community 16S rDNA PCR products both before and after blood feeding. Results are the average of three independent feedings. Black squares indicate presence of a given T-RF size, and white squares indicate absence. UF, unfed, 1, 2, 3, and 5 represent profiles at indicated number of days after a blood meal. The Mantel test (Mantel 1967, Mantel and Vanland 1970), performed using the computer program zt (Bonnet and Van de Peer 2002), showed that the similarity values between sample pairs were independent of their temporal distance after blood feeding. The order of the elements in one of two matrices was randomly relocated (10,000 permutations); one matrix consisted of Jaccard similarity values for each sample pair and the second consisted of their temporal distances (0–5 d). The observed correlation (−0.298) and associated *P* value (<0.001) strongly supported the supposition that similarity values for each sample pair are not independent of their temporal distance.

The 16S rDNA fragments obtained from site one *C. sonorensis* (Table 1) represent six taxonomic classes with 14 putative genera. Of these, eight have been isolated from other hematophagous dipterans, *Comomonas*, *Enterobacter*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Rickettsia*, *Stenotrophomonas*, and *Staphylococcus* (Demaio et al. 1996, Perira de Oliveira et al. 2001). Most of the remaining genera typically inhabit soil, plant, or bovine ruminant environments (Achouak et al. 1998, Llop et al. 1999, Maltseva et al. 1999,

Table 1. *C. sonorensis* midgut microbial 16S rDNA, site 1

Cluster	R[a]	Division, subdivision	GenBank match Genus	E value	Score	GI no.	Predicted T-RF sizes		
							AluI	HhaI	RsaI
C3-FFF5	7	Proteobacteria, gamma	*Acinetobacter*[b]	0.0E+00	904	2326328	34, 289	169, 318	
C1-5	8	Proteobacteria, beta	*Chromobacterium*	0.0E+00	835	21898817	101, 289	11, 158, 329	50, 82
C1-1	5	Proteobacteria, beta	*Comamonas*[b]	0.0E+00	952	22535357	115, 248	319	51, 390
C1-4	8	Proteobacteria, gamma	*Enterobacter*[b]	0.0E+00	963	21327114	36, 214, 247	158, 333	69, 102, 387
C2-27	1	Fibrobacteres/ Acidobacteria	*Fibrobacter*[c]	1.0E-79	303	174565	104	23	243
C2-FF4	2	Proteobacteria, gamma	*Klebsiella*[b,d]	0.0E+00	870	26000221	124		398
C1-18	1	Proteobacteria, alpha	*Paracoccus*	0.0E+00	745	13489268	210	23	382
C2-26	12	Actinobacteria	*Propionibacterium*[c]	0.0E+00	908	20750383	104, 137, 269		
C1-F8	2	Proteobacteria, gamma	*Pseudomonas*[b, d]	0.0E+00	948	23477309	210	127	38
C1-8	2	Proteobacteria, beta	*Ralstonia*	0.0E+00	950	19909106	37		441
C1-21	1	Proteobacteria, gamma	*Rahnella*	0.0E+00	950	2290270	37	335	
C2-30	6	Proteobacteria, alpha	*Rickettsia*[e]	0.0E+00	850	17298139	224–228	300	51, 95
C2-44	1	Firmicutes	*Staphylococcus*[b]	0.0E+00	969	407904	289		50
C2-39	1	Proteobacteria, gamma	*Stenotrophomonas*[b]	0.0E+00	967	8980468	247	158	50

GI number, Score, and E value represent the GenBank accession number, BLASTn similarity score, and expectation value, respectively, of the most closely related known bacterial genus to the 16S rDNA sequence cluster. Shaded areas indicate T-RFLP profiles that were present in the top 25% relative fluorescence levels of summed peak data.

[a] R represents number of 16S rDNA fragments isolated for a given cluster.

[b] Genus isolated from suborder Nematocera.

[c] Genus isolated from ruminants.

[d] Not confirmed by neighbor-joining analysis.

[e] Genus isolated from other Insecta, not Nematocera.

Saadoun 2002, Zarate et al. 2002). In the 16S rDNA clone library, the most highly represented bacterial division in *C. sonorensis* from eastern Colorado high plains was *Proteobacteria*, as summarized in Table 3. T-RFLP profiles exhibiting relative fluorescence in the top 25% for summed peak height data suggest that *Comomonas*, *Enterobacter*, *Klebsiella*, and *Chromobacterium* are among the most abundant bacterial genera for site 1. These are indicated by shaded boxes in the relevant predicted T-RF sizes in Table 1. *Alu*I-based T-RF relative fluorescence values above the mean for all site one samples indicate that *Propionibacterium* was also an abundant genus in this group of *C. sonorensis*.

The 16S rDNA fragments obtained from three midgut pools of *C. variipennis* (site 2) represent six taxonomic classes with 26 provisional genera (Table 2). Some genera were common to those found in *C. sonorensis* (Table 1), as well as other hematophagous dipterans (Demaio et al. 1996, Perira de Oliveira et al. 2001). The T-RFLP profiles showing relative fluorescence of peak heights in the top 25% are shaded (Table 2). Although *Rsa*I T-RF peak heights corresponding to *Chryseobacterium*, the most highly represented genus in the clone library, were abundant, they were generally outside the call window of >50 bp, and therefore did not allow confirmation of genus abundance by T-RFLP analysis. As with *C. sonorensis*, *C. variipennis* also carried microbiota typically found in soil, plant, and bovine ruminant habitats (Madsen 1989, Saadoun 2002, Zarate et al. 2002, Sanchez-Porro et al. 2003).

Two Sites, Two Species, Different Gut Communities. The presence/absence of T-RF peaks were matched to the predicted T-RFs for the bacterial genera identified in the cloning project (Table 4). Table 4, top, shows *C. sonorensis* midgut 16S rDNA sequenced fragments compared with the T-RF peaks of both *C. sonorensis* and *C. variipennis*. Table 4, bottom, shows *C. variipennis* presented in the same manner. The numbers indicate the frequency of informative T-RFs for one or more of the restriction enzyme digests. The data presented in this manner demonstrate how sequence analysis of small clone libraries is complemented by T-restriction fragment-length polymorphism community composition analysis.

According to Table 4, a number of similar T-RF sizes were found in samples representing geographically separated midge genera, even though the corresponding 16S rDNA sequences were not isolated during the sequencing project. *Streptomyces* and *Halomonas* (Table 4, bottom) were the only candidates which did not provide informative restriction sites for the enzyme panel chosen and therefore were not detected. In contrast, *Moraxella* and *Streptococcus* 16S rDNA fragments bore restriction enzyme sites (Table 2) but were not detected by T-RFLP, suggesting that they were below the detection limits.

To quantify midgut community compositional differences between midge species and between live-trapped and laboratory-emerged adults of the same species, both Jaccard similarity values (presence/absence) and Euclidean distances (peak heights) were

Table 2. *C. variipennis* midgut microbial 16S rDNA fragments, site 2

Cluster	R[a]	Division, subdivision	Genus species	E value	Score	Gi No.	Predicted T-RF Sizes		
							AluI	HhaI	RsaI
N2-24	1	Firmicutes	*Acholeplasma*[b]	0.0E+00	509	537897	109	191	
N2-14	1	Proteobacteria, gamma	*Acinetobacter*[c]	0.0E+00	944	6120060		170	
N2-6	1	Proteobacteria, alpha	*Afipia*	0.0E+00	850	2290235	174	307	73
N1-19	2	Proteobacteria, alpha	*Caulobacter*	0.0E+00	837	4127732	157, 222	142	50
N2-1	3	Actinobacteria	*Corynebacterium*	0.0E+00	902	665618	181	329	44-58, 90
N1-2	32	Bacteroidetes	*Chryseobacterium*[b]	0.0E+00	448	14190076	33, 56-67	23	48
N3-6	1	Proteobacteria, beta	*Delftia* sp.	0.0E+00	948	16209595	116	168	390
N1-12	1	Firmicutes	*Eubacterium*	0.0E+00	379	762812	237		47
N3-7	1	Bacteroidetes	*Flavobacterium*[b,c]	0.0E+00	440	5103296	35		58
N2-5	2	Bacteroidetes	*Flexibacter*	0.0E+00	569	14571916	170	31	80
N1-1	1	Proteobacteria, gamma	*Halomones*	0.0E+00	928	27818788	36		
N1-8	1	Actinobacteria	*Kocuria*	0.0E+00	928	2108304	33	434	418
N2-19	1	Proteobacteria, beta	*Leptothrix*	0.0E+00	936	14537937	196	29	435
N2-18	1	Proteobacteria, gamma	*Moraxella*[b]	0.0E+00	948	9743632	289	328	
N1-20	1	Actinobacteria	*Nocardioides*[d]	0.0E+00	922	22217928	269	63	46
N2-23	1	Firmicutes	*Peptostreptococcus*[e]	0.0E+00	607	454181	216		35
N2-11	2	Proteobacteria, gamma	*Pseudomonas*[c,d]	0.0E+00	908	7920706	34	162, 170	428
N1-5	5	Actinobacteria	*Propionibacterium*[e]	0.0E+00	922	21667962	136		
N2-12	1	Proteobacteria, gamma	*Psychromonas*	0.0E+00	952	15420629	37	335	414
N1-10	1	Proteobacteria, beta	*Ralstonia*[d]	3.00E−06	60	17431752		233	319
N2-13	1	Proteobacteria, beta	*Roseateles*	0.0E+00	868	3138784	115	167	433
N1-7	1	Firmicutes	*Staphylococcus*	0.0E+00	948	14009313	288	157	49
N2-10	1	Proteobacteria, gamma	*Shewanella*	0.0E+00	833	14572029	21		
N1-18	1	Firmicutes	*Streptococcus*	0.0E+00	971	2183312	36		
N1-9	1	Actinobacteria	*Streptomyces*	0.0E+00	799	4323617	179	422	
N2-7	1	Proteobacteria, beta	*Zoogloea*	0.0E+00	589	26005785	118		436

GI number, Score, and E value represent the GenBank accession number, BLASTn similarity score, and expectation value, respectively, of the most closely related known bacterial genus to the 16S rDNA sequence cluster. Shaded areas indicate T-RFLP profiles that were present in the top 25% relative fluorescence levels of summed peak data.

[a] R represents number of 16S rDNA fragments isolated for a given cluster.

[b] Genus isolated from other Insecta, not Nematocera.

[c] Genus isolated from suborder Nematocera.

[d] Not confirmed by neighbor-joining analysis.

[e] Genus isolated from ruminants.

calculated for all T-RFLP sample pairs. One-way analysis of variance indicated significant differences ($P < 0.001$) between within-species and between-species sample pairs for both Jaccard similarity values and Euclidean distances. These results were also graphically illustrated using principle components analysis (PCA) of presence/absence and peak height data (Fig. 2). Principle component 1 (PC1) represented 38.4% of the variance between laboratory-emerged and live-trapped samples, whereas PC2 represented 17% of the variance between the two species tested. Separation of the three groups suggests differences in microbial community composition between both species, as well as between laboratory-emerged and live-trapped.

Table 3. Number of 16S rDNA fragment isolates per bacterial division

Division	*C. sonorensis*	*C. variipennis*
Actinobacteria	12	11
Bacteroidetes	0	35
Fibrobacteres/Acidobacteria	1	0
Firmicutes	1	5
Proteobacteria	43	15

The 16S rDNA sequence fragments listed in Tables 1 and 2 are depicted as number of clones per bacterial division.

Terminal-Restriction Fragment-Length Polymorphism of Laboratory-Emerged Wild *C. sonorensis* Adults versus Live-Trapped Adults. To minimize the presence of environmental contaminants, *C. sonorensis* were collected at the pupal stage and allowed to eclose in the laboratory. For comparison, live adults were also collected from the same site. T-RFs corresponding to several bacterial genera were present in laboratory-emerged adults but not detected in live-trapped adults from the same collection site. For example, *Fibrobacter* and *Ralstonia* were not detected in live-trapped adult females by T-restriction fragment-length polymorphism. In addition, many genera were detected less frequently in midguts from live-trapped adults (Table 4), as corroborated by the statistical analyses described above.

Discussion

This study provides an additional demonstration of the utility of T-RFLP analysis for describing differences in complex microbial communities of insect midguts. When integrated with cloning and sequencing of independent 16S rDNA isolates, these dovetailed approaches allowed us to provisionally identify bacteria by sequence analysis of 16S rDNA fragments and to detect bacterial 16S rDNA fragments by

Table 4. Midgut pool T-RF fragments matching bacterial clusters

Genus	Cluster	*C. sonorensis*		*C. variipennis*
		Emerged wild adults	Live-trapped adults	Emerged wild adults
Source: *C. sonorensis*				
Acinetobacter	C3-FFF5	4 (4)	2 (6)	4 (5)
Chromobacterium	C1-5	4 (4)	5 (6)	5 (5)
Comomonas	16-33	3 (4)	3 (6)	4 (5)
Enterobacter	C1-4	3 (4)	2 (6)	3 (5)
Fibrobacter	C2-27	2 (4)	0 (6)	3 (5)
Klebsiella	C2-FF4	3 (4)	5 (6)	4 (5)
Paracoccus	C1-18	2 (4)	2 (6)	4 (5)
Propionibacterium	C2-26	4 (4)	5 (6)	4 (5)
Rahnella	C1-21	2 (4)	1 (6)	3 (5)
Ralstonia	C1-8	1 (4)	0 (6)	1 (5)
Rickettsia	C2-30	2 (4)	1 (6)	3 (5)
Staphylococcus	C2-44	3 (4)	2 (6)	3 (5)
Stenotrophomonas	C2-39	1 (4)	4 (6)	3 (5)
Source: *C. variipennis*				
Acholeplasma	N2-24	0 (4)	4 (6)	2 (5)
Acinetobacter	N2-14	0 (4)	2 (6)	2 (5)
Actinobacteria	N1-8	1 (4)	1 (6)	4 (5)
Afipia	N2-6	4 (4)	6 (6)	5 (5)
Caulobacter	N1-19	3 (4)	2 (6)	4 (5)
Chryseobacterium	N1-11	3 (4)	5 (6)	4 (5)
Corynebacterium	N2-1	1 (4)	2 (6)	3 (5)
Cytophagales	N2-8	2 (4)	4 (6)	5 (5)
Delftia	N3-6	4 (4)	6 (6)	4 (5)
Eubacterium	N1-12	3 (4)	0 (6)	2 (5)
Flavobacterium	N3-7	3 (4)	4 (6)	4 (5)
Flexibacter	N2-5	2 (4)	4 (6)	5 (5)
Halomonas	N1-1	0 (4)	0 (6)	0 (5)
Leptothrix	N2-19	4 (4)	1 (6)	4 (5)
Moraxella	N2-18	0 (4)	0 (6)	0 (5)
Nocardiodes	N1-20	4 (4)	1 (6)	1 (5)
Peptostreptococcus	N2-23	1 (4)	1 (6)	2 (5)
Propionibacterium	N1-5	4 (4)	5 (6)	4 (5)
Pseudomonas	N2-21	0 (4)	2 (6)	3 (5)
Psychromonas	N2-12	2 (4)	1 (6)	3 (5)
Roseateles	N2-13	4 (4)	4 (6)	5 (5)
Shewanella	N2-10	4 (4)	3 (6)	3 (5)
Streptococcus	N1-18	0 (4)	0 (6)	0 (5)
Streptomyces	N1-9	0 (4)	0 (6)	0 (5)
Zooglea	N2-7	4 (4)	1 (6)	4 (5)

Cloned 16S rDNA sequences were assigned a putative bacterial genus based on BLASTn analysis (Tables 1 and 2) and predicted T-RFLP profiles (TRFSEQ software). T-RFLP profiles for each field sample were obtained after digestion with restriction enzymes *Hha*I, *Rsa*I, or *Alu*I. Each column indicates the frequency of field samples which displayed the predicted T-RFLP pattern (≥50 bp) for one or more restriction digests within ±2 bp of the predicted TRF. Parentheses denote the total number of midgut pools assayed for each group.

T-RFLP that were not sequenced in the clone library. These T-RFLP analyses demonstrate significant differences in the gut bacterial communities of two *Culicoides* spp. populations from geographically separated sites. Whether the differences in midge gut bacterial communities reported here will be maintained between *Culicoides* spp. in other habitats, or will eventually be correlated with vector competence, requires further experimentation.

The colony feeding study demonstrated that the bacterial community composition of the midge gut shifts after a blood meal. Profiles of T-RFs detected in unfed females before the feeding disappeared, and different T-RFs occurred in the days after the blood meal. The latter group may represent rare community members, exploiting the introduction of a high protein nutrient source. For a given T-RF to be detected, it must represent at least 0.5–1.0% of the 16S rDNA sequence population of a given sample. Because only a single restriction enzyme was used in the colony feeding study, unambiguous bacterial genera assignments could not be made. These limitations point to the importance of careful selection of multiple restriction enzymes in any T-restriction fragment-length polymorphism study in which preliminary bacterial identification is required. The detection of post-blood meal microbiotic shifts in this study demonstrates the utility of this method for future assessment of midgut microbiota shifts under differing conditions pertinent to vector competence.

The T-RFs detected in unfed colony females (UF) that disappeared after a blood meal may represent microbiota retained in the pupal meconium of unfed female midges or, alternatively, an overabundance of microbiota carried over from an undefined larval rearing medium (unpublished data). For the field study, laboratory-emerged wild adult females were used in an effort to minimize the introduction of environmen-

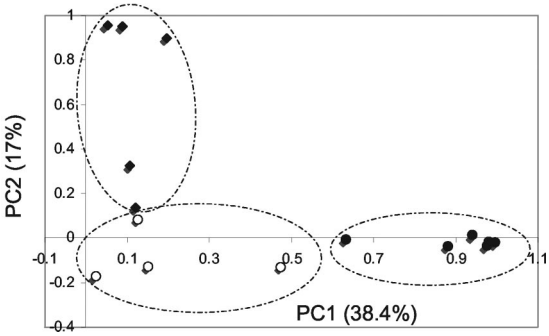

Fig. 2. PCA (SPSS 9.0). T-restriction fragment-length polymorphism presence/absence and peak height data for all three restriction enzyme (*Alu*I, *Hha*I, and *Rsa*I) data sets were analyzed. Open and filled circles represent *C. sonorensis* laboratory-emerged wild adults and live-trapped adults, respectively. Diamonds represent *C. variipennis* laboratory-emerged wild adults. The percentage of variance explained by each PC is in parentheses. PC1 (eigenvalue, 5.80) represents the variance between laboratory-emerged and live-trapped adults, whereas PC2 (eigenvalue, 2.63) represents the variance between the collection sites. Each group is encircled with an ellipse.

tal contaminants. Interestingly, there were differences in T-RF peak heights between laboratory-emerged and live-trapped *C. sonorensis* from the same collection site (data not shown). This evidence suggests that, in addition to qualitative differences, there are also quantitative differences in the midgut bacteria of these two groups. Together, these data suggest that the pupal meconium is not passed concurrently with adult midge emergence, as has been seen for other nematocerans, such as mosquitoes (Moll et al. 2001); however, further experimentation will be required to confirm this hypothesis.

Interestingly, bacterial genera found in other hematophagous dipterans were represented among both midge species (Seitz et al. 1987, Maudlin et al. 1990, Vasanthi and Hoti 1992, Demaio et al. 1996, Fouda et al. 2001), because some bacteria may facilitate digestion of the blood meal (Fouda et al. 2001). In addition, at least one genus is a candidate for genetic engineering, as *Caulobacter*, an adherent aquatic bacteria, has been genetically engineered to carry mosquitocidal toxins for larval biocontrol (Thanabalu et al. 1992).

Other studies have suggested that variation in midgut bacterial species affects the establishment of pathogens vectored by biting dipterans. For example, *Rickettsia* has been implicated in the modulation of pathogen transmission in tsetse flies (Maudlin et al. 1990) and has been previously isolated from *Culicoides* (Hertig and Wolbach 1924). Although a rickettsial 16S rDNA was isolated in the orbivirus competent *C. sonorensis* population of eastern Colorado, T-restriction fragment-length polymorphism analysis suggested that it was also present in the Nebraska *C. variipennis* population. Still, the possibility remains that more subtle factors, such as genetic variation between closely related bacterial species or synergy between midgut bacterial community members may contribute to the phenotypic variation observed between vector insect populations.

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